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Molecular Detection of Hepcidin (HAMP) Gene in Selected Iron Deficiency Anemia Patients from Basrah Governorate, Iraq

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Keywords
Anemia, protein, polymorphisms, amplified

Abstract:
Anemia constitutes one of the most common blood diseases, which can be dangerous and more complex than it appears. This danger comes from the apparent lack of iron which is a vital element in various metabolic and anabolic processes. The HAMP gene produces a protein called Hepcidin, which has a very important role in iron metabolism. This study aimed at the molecular detection of HAMP gene in selected iron deficiency anemia patients, as well as healthy control, from Basra community in the south of Iraq. Forty five samples were collected from private clinics, which were divided into 33 samples for affected patients and 12 samples represented the control group. A450 bp fragment containing HAMP exon 3was amplified using Forward: CCGTTCCCTGCTCACATTCC and Reverse: TTCACCAACTTTCCTGGCAAC . Eight different polymorphisms were obtained depending on the number of mutations that occurred for the gene compared to what was recorded in the GenBank for the same gene, 6 polymorphisms, studies represented the affected and 2 represented the control, they were all registered in the GenBank under accession numbers LC707242, LC707243, LC707244, LC707245, and LC707246 (patients' samples), and LC707247, and LC707248,LC707249 (control samples). The polymorphisms obtained in the current study had a number of different mutations, whether silent or missense, some mutations occurred in more than one polymorphism, while some occurred in one polymorphism. It was noted that some mutations occurred in all polymorphisms of the study. When conducting a BLAST analysis, it was found that the results obtained were closer to each of the genes recorded in America and South Africa, and this can be clearly observed in the analysis of the phylogenetic tree. The results of the analysis of the three-dimensional structure of the expected protein indicated a great match between the polymorphisms of the study except for polymorphism LC707245. As a result of the occurrence of these mutations, the HAMP gene in Iraq has more than one polymorphism, these polymorphisms may be associated with the function of the gene. Therefore, further studies are needed to link this polymorphism to various traits associated with anemia.

Introduction:

Anemia is the most common hematologic disorder, iron deficiency being the leading cause worldwide (Elstrott et al., 2020). Often, anemia is the presenting sign of a more serious underlying condition that, if left untreated, can generate consequent morbidity (Portugal-Nunes et al., 2020).

The HAMP gene is encoding protein called Hepcidin which plays a main role to the metabolism of iron via banning the shot of iron from intestinal cells and macrophages (Melis et al., 2008). Several studies indicated that the obstruction of HAMP gene role will lead to an increase in iron load (Xu et al., 2021), while in common cases Hepcidin prevent surplus iron absorption in intestinal mucosa and maintains its

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normal level in the body (Ganz 2011). On the other hand have indicated that the activity of the Hamp gene is related to its different polymorphisms (Ganz 2006). The deficiency of hepcidin causes increase in hemochromatosis, and hepcidin excess may cause iron deficiency, iron-restricted erythropoiesis and anemia due to some mutations in the HAMP gene (Kanwar andKowdley 2013). Pandey et al., (2018) also indicated that the occurrence of mutations in the HAMP gene could lead to beneficial effect for some diseases like iron deficiency anemia, while it may be harmful for others particularly those associated with iron overload. So the HAMP genotyping is useful for the decision of treatment and getting rid of iron overload.

The HAMP gene encodes hepcidin, an antimicrobial peptide and key iron regulatory hormone. Hepcidin is mainly produced by the liver during conditions of high iron, infection, or inflammation. Hepcidin controls plasma iron levels by binding to the iron exporter ferroportin (SLC40A1; 604653) and inducing its degradation. By decreasing plasma iron levels, hepcidin provides an iron-restricted internal environment inhospitable to microbes, thereby contributing to innate immunity (Malerba et al., 2020). In humans, the HAMP gene is located on the chromosome19, consist of three exons and two introns (Kemnaet al., 2008).

Aim of the study

In the absence of previous similar studies that deals with this gene in Iraq, this study aimed to characterize the HAMP gene in Basrah Governorate, south of Iraq, in selected sample of objects with and without iron deficiency.

Materials and Methods

This study was conducted from May 2021 to June 2022, in Basrah Governorate, southern Iraq (30.536242°N 47.815819°E).

Inclusion criteria:

Patients with microcytic hypochromic anemia, low serum iron, ferritin, and transferrin saturation, and high TIBC and hepcidin, who attended private outpatient clinics.

Exclusion criteria:

Any patient with any type of anemia other than IDA and did not achieve the criteria above.

Control subjects:

Normal volunteers who have normal iron study and serum hepcidin level.

Sampling:

Forty five samples were collected (33 samples patients and 12 normal control) Three ml of blood was collected from the samples under study in EDTA tubes, then were kept in the freezer until the DNA was extracted.

The Extraction of DNA:

DNA has been extracted according to the method mentioned by Ngole et al., (2022), then the Nano drop has been used to determine the purity and concentration of DNA. It was taken into account that the ratio of 260/280 nm is close to or equal to 1.8.

The design of primer and the amplification of PCR:

The primers were designed according to Yu et al., (2012) for HAMP gene exon 3, Forward: CCGTTCCCTGCTCACATTCC and Reverse: TTCACCAACTTTCCTGGCAAC. The amplification of PCR was done according Parajes et al., (2010) in gross volume25 µl with 9.5 µl water nuclease free, 2 µl of DNA template (100ng/ml), 0.5 µl (10 µM) of forward primer, 0.5 µl (10 µM) of reverse primer and 12.5 µl of master mix, the PCR conditions were briefed in Table (1). By using 1.5% Ethidium Bromide 0.5 µg/ml-stained agarose gel the PCR product has been detected. A DNA ladder of size 2000 bp was used. Then the PCR product was purified by using Qiagen kit (Germany) QIAquick®.

The analysis of Sequences:

The sequences were analyzed in first BASE laboratory (APICAL) Malaysia. To match the resulting sequences of current study with HAMP gene in Gen Bank, the BLAST and Multiple Sequence Alignment have been carried out (Boratyn et al., 2019), depending on the highest match accession numbers have been selected (AD000684 in UAS and FM242488 South Africa).

The analysis of phylogenetic tree:

By using Mega-11 (Tamura and Kumar 2021) the phylogenetic tree has been done compared with gene sequences in both USA (NG_011563) and South Africa (AJ715525).

The 3D structure of protein:

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To detect the 3D structure of protein of resulting sequences, the SWISS-MODEL (Waterhouse et al., 2018) has been used.

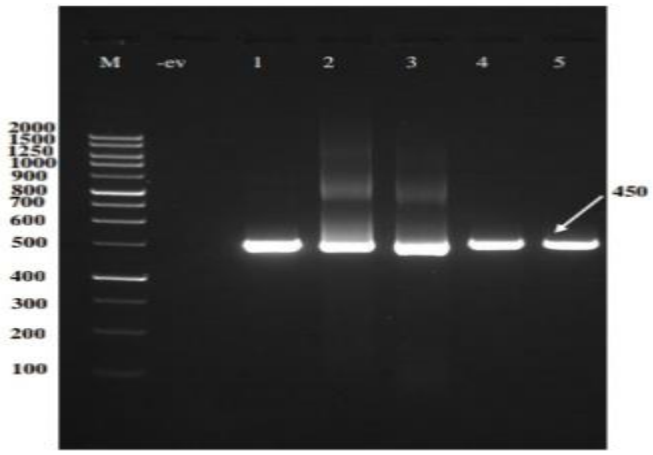
Table1: Cycling protocol and temperature of PCR amplification

Cycle step	Temp (° C)	Time	Number of Cycles
Initial Denaturation	95	5 min	1
Denaturation	94	30 s	30
Annealing	54	30 s	
Extension	72	2 min	
Final Extension	72	10 min	1

Results:

Analysis of HAMP polymorphisms

The size of the PCR product was 450bp (Figure 1).Compared with what was recorded in the Gen Bank, eight polymorphisms of the HAMP gene were obtained in Basrah Governorate - southern Iraq, they were all registered in the Gen Bank with accession numbersLC707242 (5 samples), LC707243 (6 samples), LC707244 (7 samples), LC707245(6 samples),LC707246(3 samples),LC707247(6samples),and control group LC707248(7samples),LC707249(5sampls) , (Figure 2), which accession number of USA (NG-011563) and South Africa (AJ715525), the highest percentage of match with them. All mutations in the eight polymorphisms were summarized in Table 2. A different number of mutations occurred in each polymorphism, some mutations occurred only in patient samples, others occurred in control samples only, and some to a lesser extent occurred in the polymorphisms of patients and the control.



(Figure 1)

The results of the electrophoresis of a segment of the HAMP gene showed the appearance of bundles of bp nucleotide pair size (450) between the two sites(69-274) using a primer designed for the first time in the current study by primer 3 program(M:represent DNA Ladder2000bp,80 Volute,45minutes)

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	No.	Reference Gene		polymorphism	Position	Mutations	
		NG_011563 USA	AJ715525 South Africa			Type	Amino acid
Polymorphism (LC707242)	1	G	G	C	36	Silent	
	2	G	G	T	82	Missense	Cysteine to glycine
	3	G	G	A	96	Silent	
	4	A	A	C	179	Missense	Serine to tyrosine
	5	C	C	A	180	Missense	Serine to tyrosine
	6	C	C	G	181	Missense	Leucine to Valine
	7	C	C	A	292	Missense	Leucine to isoleucine
	8	T	T	A	297	Silent	
	9	G	G	T	377	Missense	Glycine to Valine
	10	A	A	G	413	Missense	Lysine to Arginine
Polymorphism (LC707243)	1	C	C	T	3	Silent	
	2	G	G	C	36	Silent	
	3	G	G	A	96	Silent	
	4	T	T	A	118	Missense	Phenylalanine to isoleucine
	5	A	A	C	148	Missense	Arginine to Serine
	6	C	C	A	192	Silent	
	7	T	T	G	269	Missense	Phenylalanine to Cysteine
	8	T	T	G	382	Missense	Serine to Arginine
	9	A	A	G	448	Missense	Asparagine to aspartic acid
Polymorphism LC707244)	1	C	C	T	3	Silent	
	2	C	C	T	70	Missense	Serine to Proline
	3	T	T	A	240	Silent	
	4	G	G	T	326	Missense	Leucine to Cysteine
	5	T	T	A	327	Missense	Leucine to Cysteine
	6	T	T	C	404	Missense	Serine to Leucine
	7	C	C	T	432	Silent	
Polymorphism LC707245)	1	T	T	A	57	Silent	
	2	T	T	C	126	Silent	
	3	C	C	A	192	Silent	
	4	T	T	C	246	Silent	
	5	A	A	G	247	Missense	Lysine to Valine
	6	A	A	T	248	Missense	Lysine to Valine
	7	G	G	T	337	Missense	Serine to Alanine
	8	G	G	A	398	Missense	Glycine to glutamic acid
	1	C	C	G	22	Missense	Proline to valine

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Polymorphism LC707246)	2	C	C	T	23	Missense	Proline to valine
	3	G	G	A	96	Silent	
	4	C	C	A	192	Silent	
	5	A	A	C	233	Missense	Histidine to Proline
	6	G	G	A	237	Silent	
	7	A	A	T	273	Missense	Glutamine to Histidine
	8	C	C	G	431	Missense	Proline to Araginine
	9	C	C	T	450	Silent	
Polymorphism (LC707247)	1	T	T	C	5	Missense	Leucine to Proline
	2	C	C	A	58	Missense	Proline to Serine
	3	C	C	G	59	Missense	Proline to Serine
	4	G	G	A	96	Silent	
	5	G	G	T	149	Missense	Serine to Isoleucine
	6	T	T	C	208	Silent	
	7	A	A	T	235	Missense	Arginine to tryptophan
	8	G	G	T	377	Missense	glycine to valine
	9	T	T	C	447	Silent	
Polymorphism LC707248)	1	C	C	T	3	Silent	
	2	C	C	T	42	Silent	
	3	T	T	A	57	Silent	
	4	T	T	C	171	Silent	
	5	A	A	G	249	Silent	
	6	T	T	A	336	Silent	
	7	T	T	C	416	Missense	Isoleucine to Threonine
	8	T	T	A	417	Missense	Isoleucine to Threonine
Polymorphism LC707249)	1	C	C	T	3	Silent	
	2	C	C	T	42	Silent	
	3	T	T	A	57	Silent	
	4	T	T	C	159	Silent	
	5	C	C	A	192	Silent	
	6	T	T	G	222	Silent	
	7	T	T	A	295	Missense	Serine to threonine
	8	T	T	A	336	Silent	
	9	G	G	A	402	Silent	

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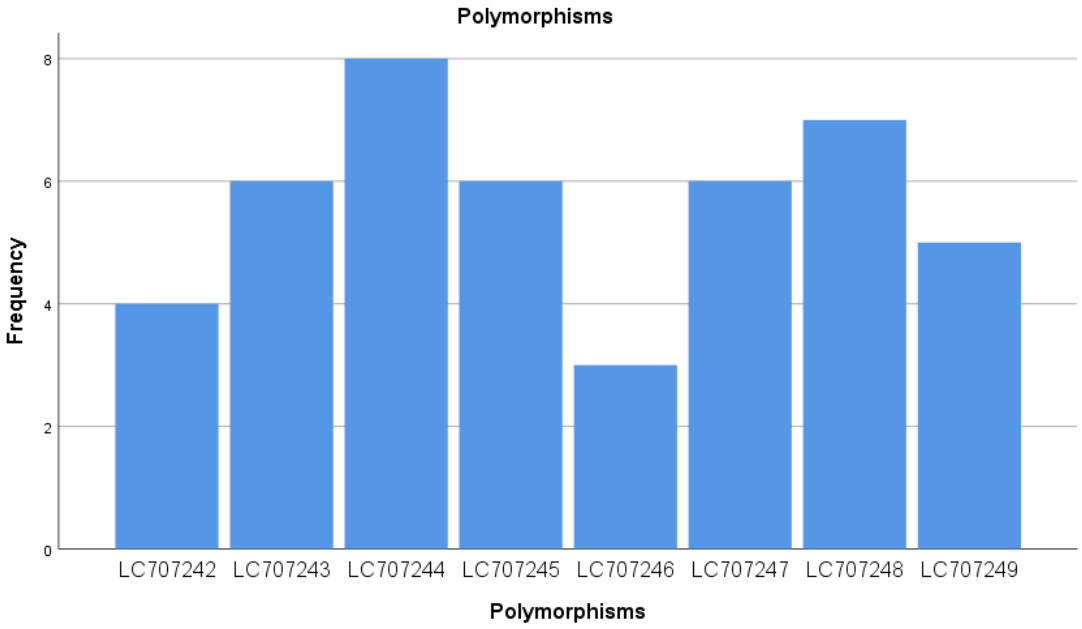
	10	T	T	C	416	Missense	Isoleucine to Threonine
	11	T	T	A	417	Missense	Isoleucine to Threonine

Table 3 HAMP Gene Polymorphisims

polymorphism	Frequency	Percent%	OR(95%Cl)	P.value
LC707242	4	8.7	0.7805(0.1964-3.1182)	0.7258
LC707243	6	13.3	1.2308(0.3470-4.3657	0.7479
LC707244	8	17.4	1.7297(0.5192-5.7630)	0.3722
LC707245	6	13.9	1.2308(0.3470-4.3630)	0.7479
LC707246	3	6.7	0.5714(0.1281-2.5493)	0.4633
LC707247	6	13.3	1.2308(0.3470-4.3657)	0.7479
LC707248	7	15.6	1.4737(0.4305-5.0446)	0.5368
LC707249	5	11.1		
Total	45	100.0		

Table 3 :the frequency of polymorphisms



The current study found that the segment that was amplified from the *hamp* gene(exon3) contained13 mutations all of which were of the substitution type all are pre-record ,and were as follows:

1- Substitution of the adenine nitrogen base in the source sequence(USA snd South Africa)to an cytocine in the site study sample sequenc ,c.36G>C ,the mutation was silent and did not change the type of amino acid.

2- The substitution guanine to thymine in at sites c. 82G>T,c.326,c.337, c.377
lead to translocation of the amino acid cytosine to glycine.

3- While replacing the guanine to adenine at the sites c.96G>A,398,237,402,and this mutation was silent.

4- Adenine base converts to cytosine at the followng sites c.179A>C, 148,233)it leads to conversion of the amino acid serine to tyrosin in situ179, Arginine to Serine in 148 and Histidin to proline in situ 233

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5- The replacement of cytocine with Adenine at sites 180,292,192,58 resulted in the change of the amino acid serine to Tyrosine in situ 180,Leucine to isoleucine in 292,while it was silent on site 192,and it was convert Proline to Serine in situ 58.
6- Substituted Thymine for adenine at the base sit (297,118,240,57,336,417),where it was silent in the following sites(297,240,57,336),While it changed the Phenyalanine to isoleucine in situ 118,and Isoleucine to Threonine at 417
7- A>G at sites 413,247,448,249 it was convert Lysine to Arginine in sit 413,Lsine to Valine in sit 247,Asparagine to aspartic acid in situ 448and it was silent at 249.
8- C>T at sites 3,70,23,450,42 and it was silent on sites 3,432,42, and 450,While it changed Serine to Proline in position 70,Proline to Valine in position23.
9- T>G where at site 269 it caused the change of the amio acid Phenylalanine to cystine,while the mutation was silent at site 382
10- T>C at sites 404,5, 126,246,,446,416,159,5,208,447,it was convert Serine to Leucine in site 404,Leucine to Proline in sit 5, while it was silent at sites 246,208,447,416,159
11- C>G lead to an amino acid change ,Leucine to Valine at 181,Proline to Valine at 22 ,Proline to Serin at 59 and Proline to Histidin at 431.
12-A>T where replaced Lysine to Valine at site 248,Arginine to tryptophan in site 235,Glutamine to Histidine in site 273.
The analysis of phylogenetic tree (Figure 3) showed (depending on most complex probability method)a high affinity for polymorphism LC707248 and genotype LC707249, respectively(noting that both represent control samples), while the polymorphism LC707248 was farthest from the global polymorphisms.
In spite of the convergence of the 3D structure of the protein for all polymorphisms in the current study, there are differences in the 3D structure expected (Figure 2) as a result of the different mutations.

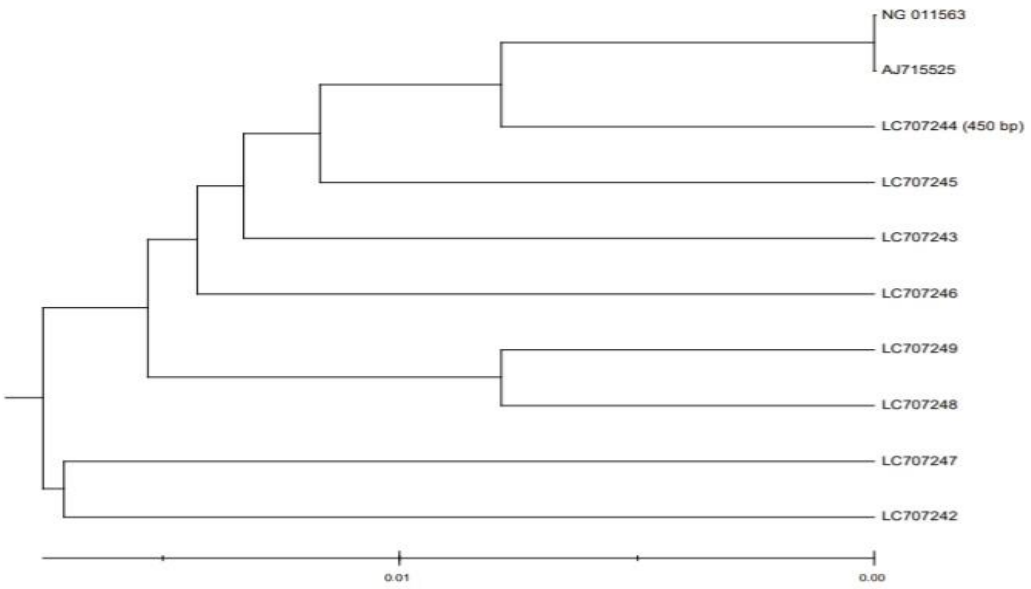

Figure 3: The Phylogenetic tree of HAMPgene in Iraq as well as USA and South Africa (LC707242, LC7707243, LC707244 LC707245, LC707246, LC707247,LC707248 and LC707249: HAMP gene in Iraq), (NG_011563: HAMP gene in USA). (AJ715525: HAMP gene in South Africa).

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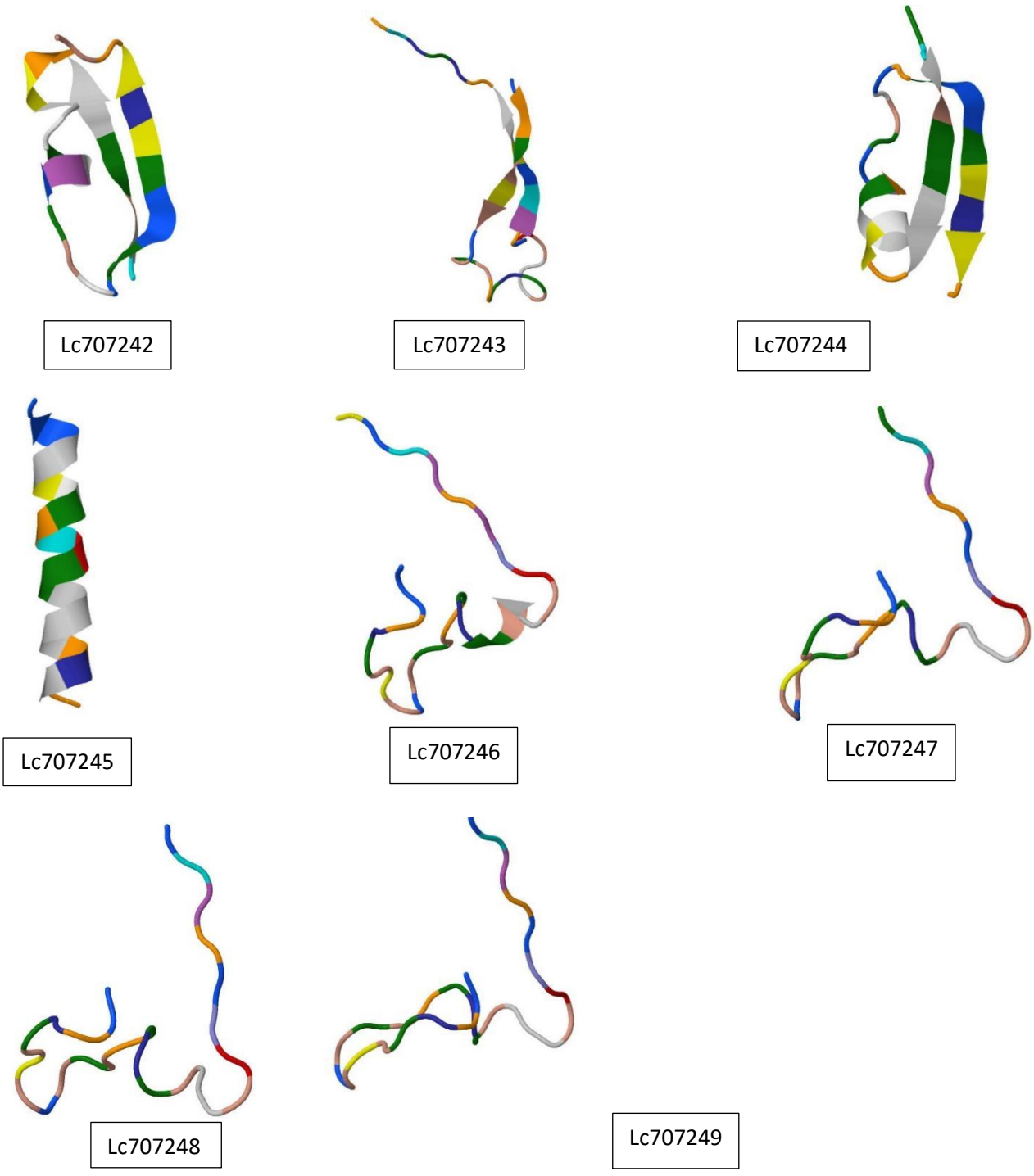

Figure 4: The 3D structure of protein of HAMP gene in Iraq

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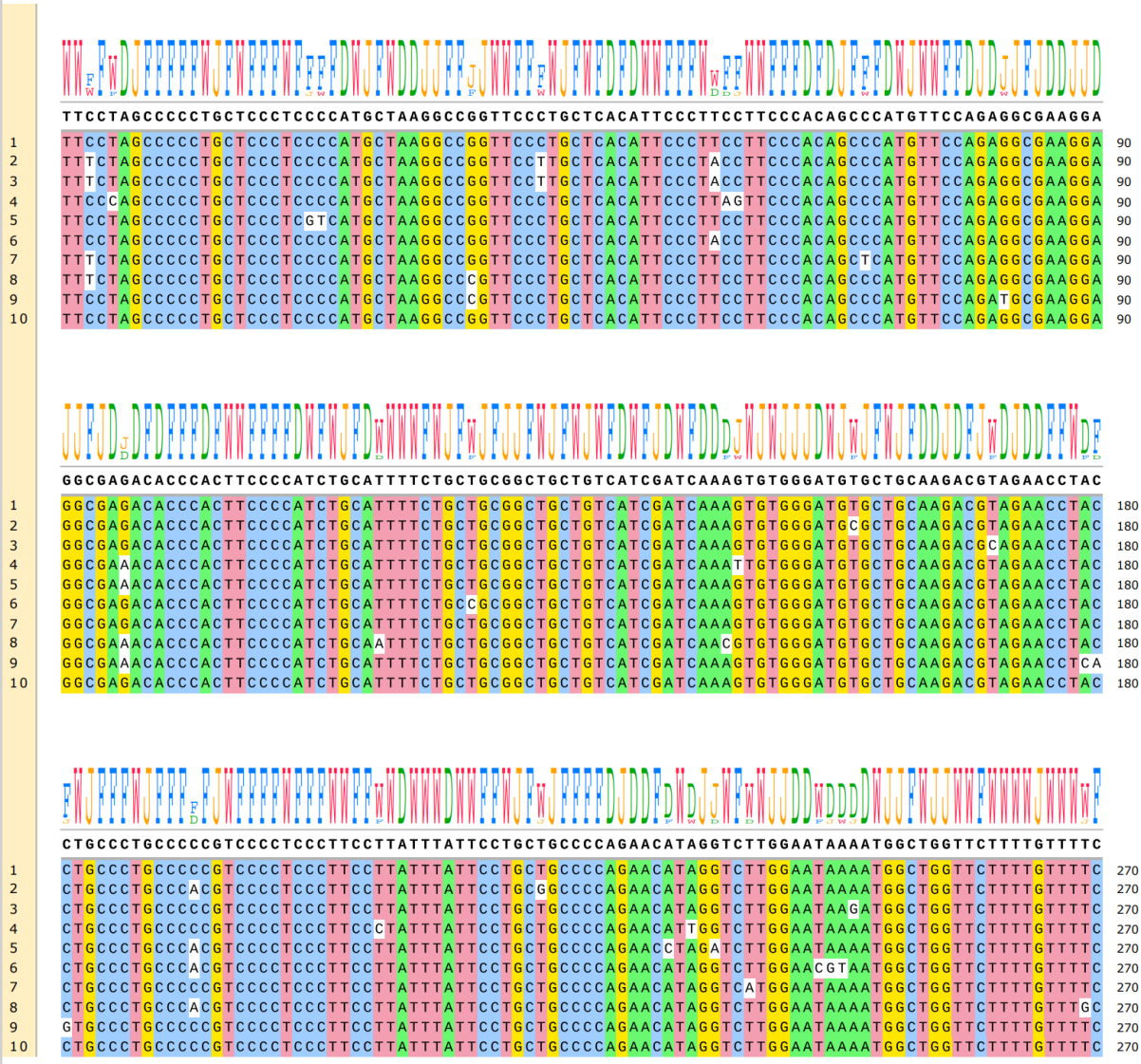
Aligned using an external algorithm

Consensus

1. NG 011563
2. LC707249
3. LC707248
4. LC707247
5. LC707246
6. LC707245
7. LC707244
8. LC707243
9. LC707242
0. AJ715525



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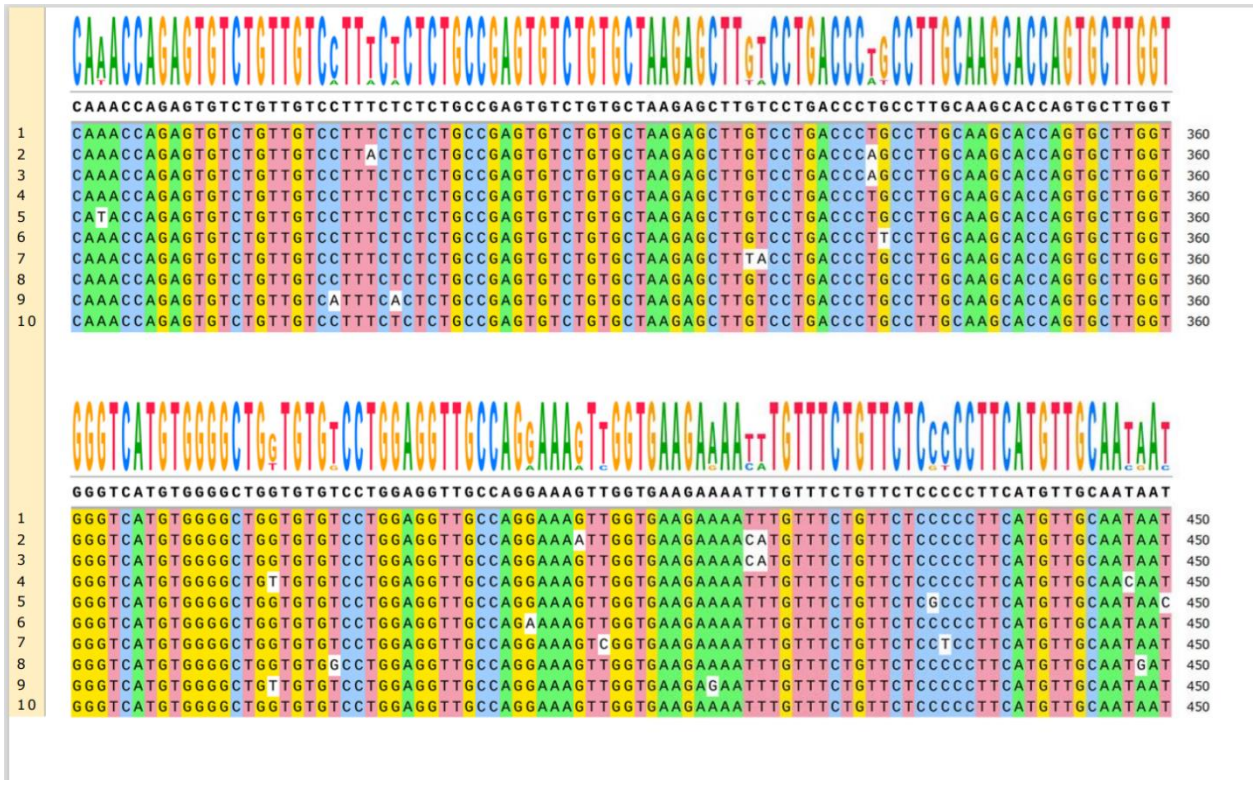

Figure 5: The Multiple Sequence Alignment for each of the USA and South Africa polymorphisms, in addition to the polymorphisms of the current study. 2, 3, 4, 5, 6, 7 ,8and 9: Iraqi polymorphisms, LC707249, LC707248, LC707247, LC707246, LC707245, LC707244, LC707243 and LC707242 respectively. 1: USA polymorphism, NG_011563. 10: South Africa polymorphism, AJ715525.

Discussion:

The results of current study agreed with McGregor (2009) about the size of PCR product, (Note that the size of the PCR product in the current study was adopted in order to give a clearer perception of the gene, given that this study is, up to our best knowledge, the first that deals with the study of gene sequences of HAMP 1 in Iraq. The results of this study are in agreement with previous studies on the possibility of polymorphisms of the HAMP gene (McLachlanet al., 2017; Abdelrhman et al., 2020;Jallow et al., 2020). The frequency of some mutations in more than one polymorphism may be due to the fact that the samples were collected from people of similar or common ancestry (Meyerson et al., 2020). The occurrence of silent mutations can have an effect as it can cause a change in the structure of the protein and thus directly affect its work as well as it can affect the folding of the protein, which can lead to instability of the protein function and affect the interaction with other biological molecules (Peyton 2021). Silent mutations affect related proteins by altering the transcription process and the accuracy and efficiency of mRNA splicing (Komar 2007).On the other hand, the effects of missense mutations come from being caused by a change in amino acids, thus, it can negatively affect the function of the protein or even positively in other cases (Khayat et al., 2021), in other words, these mutations may be the main cause of diseases (Zhang et al., 2012), or, on the contrary, it may contribute to disease resistance(Sun et al., 2021). This difference in effect may be due to the difference in the characteristics of the same amino acids, if there is often a difference (even if a little) between the amino acid before the mutation and the amino acid resulting from the occurrence of the mutation (Mohajeri and Ashrafi 2011),hence the change in the properties of the protein itself.

The affinity between the LC707248 and LC707249 polymorphisms in the phylogenetic tree analysis may

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be explained by the fact that they are the control samples, however, they were the most closely related to the global polymorphisms that were studied which could lead us to the fact that the infected samples in Iraq that were diagnosed in the current study had a number of mutations that clearly affected the gene, which made them genetically far from what exists in the world and thus this could affect negatively or positively on the activity of the protein that It is produced by the gene, and this requires us to study more broadly, linking the polymorphisms obtained in the study with the different characteristics that are directly or indirectly affected by the work of the gene (Zhang et al., 2019).

The clear convergence in the expected protein 3D structure was in all the polymorphisms obtained in the study,(patient and control) but the LC707245 polymorphism (although it represents patents samples) …….

Conclusion and recommendations:

The results of this study clearly show the presence of more than one polymorphism of the HMP gene in the sample tested. Those polymorphisms may be considered regarding gene function; therefore, it is necessary to conduct more detailed studies on the relationship of the polymorphisms of the gene to the different physiological characteristics, as well as pathological conditions related to iron metabolism, especially since the studies that dealt with this gene in Iraq are very few.

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